Development of a Sensitive Peptide-Based Immunoassay: Application to Detection of the Jun and Fos Oncoproteins†

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Received November 28, 1995; Revised Manuscript Received April 22, 1996⊗

ABSTRACT: c-Jun and c-Fos belong to the bZIP class of transcriptional activator proteins, many of which have been implicated in the neoplastic transformation of cells. We are interested in engineering dominant-negative leucine zipper (LZ) peptides as a means of sequestering these proteins *in vivo* in order to suppress their transcriptional regulatory activity. Toward this end, we have developed a novel immunoassay for measuring the dimerization affinities of dimeric Jun and Fos complexes. This peptide-based ELISA relies on the fact that Jun and Fos preferentially form heterodimers via their leucine zipper domains. Recombinant Jun leucine zipper peptides (either native JunLZ or a V36→E point mutant) were labeled with biotin and specifically bound through a leucine zipper interaction to a FosLZ−glutathione *S*-transferase fusion protein adsorbed onto the wells of an ELISA tray. Jun:Fos complexes were subsequently detected using a recently developed streptavidin-based amplification system known as enzyme complex amplification [Wilson, M. R., & Easterbrook-Smith, S. B. (1993) *Anal. Biochem. 209*, 183−187]. This ELISA system can detect subnanomolar concentrations of Jun and Fos, thus allowing determination of the dissociation constants for complex formation. The dissociation constant for formation of the native JunLZ:FosLZ heterodimer at 37 °C was determined to be 0.99 ± 0.30 nM, while that for JunLZ(V36E):FosLZ heterodimer was 0.90 ± 0.13 μM. These results demonstrate that the novel peptide-based ELISA described herein is simple and sensitive and can be used to rapidly screen for potential dominant-negative leucine zipper peptides.

c-Jun and c-Fos are members of the basic-region leucine zipper (bZIP) family of eukaryotic transcription factors which bind to DNA either as homo- or heterodimers. Dimerization of bZIP proteins, which is a prerequisite for binding to their cognate DNA enhancer elements, is mediated by a leucine zipper (LZ)[1] domain (Landschulz *et al*., 1988). The LZ domain comprises a coiled-coil of parallel α-helices (Crick, 1953), one contributed from each monomer, which wrap around one another with a slight superhelical twist (O'Shea *et al*., 1991; Junius *et al*., 1996). This coiled-coil interaction juxtaposes the adjacent basic-region DNA-binding domains of each monomer such that they can each bind to one half-site of a dyad-symmetric DNA recognition sequence (Ellenberger *et al*., 1992; König & Richmond, 1993; Glover & Harrison, 1995).

c-Jun and c-Fos regulate the transcription of numerous genes which are important in cell growth and differentiation. Their ability to transduce afferent growth signals into specific genetic responses means that they represent a critical nexus between normal and aberrant cell growth. Not surprisingly, therefore, abnormal regulation and/or expression of these proteins has been implicated in the neoplastic transformation of cells (Angel & Herrlich, 1994). Since the LZ interaction modulates the range of heterodimeric and homodimeric bZIP proteins which are present in the cell nucleus, there has been considerable effort directed toward understanding the molecular interactions which govern the affinity, specificity, and kinetics of LZ formation (O'Shea *et al*., 1992; Thompson *et al*., 1993; Graddis *et al*., 1993; Monera *et al*., 1994; Zhou *et al*., 1994; Kohn *et al*., 1995; Lumb & Kim, 1995; Wendt *et al*., 1995). It is anticipated that such information might facilitate the rational design of dominant-negative LZ domains or other agents which are capable of blocking the activity of bZIP proteins which have been implicated in oncogenic transformation (John *et al*., 1994).

One problem with such studies lies in the need to measure the dimerization affinities of native and engineered LZ domains, since the dissociation constants are typically lower than the lower limit resolvable using analytical ultracentrifugation ($K_d \approx 10^{-7}$ M). While dissociation constants have been measured for JunLZ:FosLZ complexes using fluorescence techniques (Pernelle *et al*., 1993; Patel *et al*., 1994), we set out to design a simple and sensitive immunoassay system that could be used not only to measure Jun and Fos concentrations in, for example, neoplastic tissue, but which could also be used to rapidly screen dominant-negative Jun and Fos LZ domains. We describe here a novel, peptide-

† This work was conducted as part of the Australian Commonwealth Government's Cooperative Research Centre for Molecular Engineering & Technology: Sensing and Diagnostic Technologies. This work was supported by research grants to S.B.E. and G.F.K. from the CRC for Molecular Engineering & Technology, and to G.F.K. and A.S.W. from the National Health and Medical Research Council of Australia and the University of Sydney Cancer Research Fund. J.P.M. and K.H.H. were supported by a Postdoctoral Research Fellowship and an Australian Postgraduate Award, respectively, from the Australian Research Council.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: b, biotinylated; bCys, cysteine biotinylation; bLys, lysine biotinylation; bZIP, basic-region leucine zipper; ECA, enzyme complex amplification; GαHRP, goat anti-HRP polyclonal antibody; GST, glutathione *S*-transferase; HDC, heat-denatured casein; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; IAA, iodoacetamide; IPTG, isopropyl β-D-thiogalactopyranoside; LZ, leucine zipper; SA, streptavidin.

S0006-2960(95)02817-0 CCC: $12.00

based ELISA which relies on plate-bound LZ domains rather than antibodies to "capture" Jun and Fos. Detection of plate-bound antigen was achieved using the recently developed enzyme complex amplification (ECA) system (Wilson & Easterbrook-Smith, 1993), which permitted a straightforward quantitation of subnanomolar concentrations of Jun and Fos and the measurement of nanomolar dissociation constants.

MATERIALS AND METHODS

Materials. Dithiothreitol, *S*-hexylglutathione-agarose, human and bovine thrombin, and iodoacetamide (IAA) were obtained from Sigma-Aldrich Pty Ltd. (Sydney, NSW, Australia). Isopropyl β-D-thiogalactopyranoside (IPTG) was from Progen Industries Ltd. (Darra, Qld, Australia). Chromatography-grade acetonitrile was from Rhône-Poulenc Laboratory Products (Clayton, Vic. Australia). Streptavidin (SA) and horseradish peroxidase (HRP) were obtained from Boehringer Mannheim Australia Pty Ltd. (Castle Hill, NSW, Australia). SA conjugated to HRP was from Silenus Laboratories (Hawthorn, Vic., Australia). Goat anti-HRP (GαHRP) was supplied by Jackson Immuno Research Laboratories Inc. (Westgrove, PA). Biotin-XX-NHS was supplied by Calbiochem (San Diego, CA). Iodoacetyl-LC-biotin was obtained from Pierce (Rockford, IL).

Cloning of the fosLZ Gene. A *fosLZ* gene was designed and constructed so that the encoded peptide was compatible with a recombinant JunLZ (rJunLZ) peptide which we developed previously (Riley *et al*., 1994). The core c-FosLZ sequence (LTDTLQAETDQLEDEKSALQTEIANLLKEK-EKLEFILAA) was bracketed by a Tyr residue at the C-terminus to facilitate spectrophotometric quantitation and a Met-Cys-Gly-Gly linker at the N-terminus to allow the formation of disulfide-linked dimers (O'Shea *et al*., 1989). This peptide sequence was back-translated to generate the corresponding DNA sequence using only those codons commonly found in highly expressed *Escherichia coli* genes. A synthetic gene (*fosLZ*) based on this DNA sequence was designed and constructed by annealing and subsequently ligating four single-stranded oligonucleotides with a 13-base-pair central overlap (Riley *et al*., 1994). The synthetic gene was cloned into the pGEX-2T plasmid to enable expression of FosLZ peptide as a fusion with *Schistosoma japonicum* glutathione *S*-transferase (GST) protein (Smith & Johnson, 1988). The final plasmid clone (pFLZ) was confirmed by DNA sequencing.

A V36→E mutation was introduced into the pJLZ plasmid using the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA), which is based on the method of Deng and Nickoloff (1992). The unique *Pst*I restriction site in the pJLZ plasmid was removed during the mutation procedure using a selection primer, thus allowing selection of candidate mutant clones by *Pst*I restriction digestion. The final clone was confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins. The fusion proteins rFosLZ-GST and rJunLZ-GST were overexpressed and purified in soluble form using GSH affinity chromatography, as described previously (Riley *et al*., 1994). The isolated zipper peptides rJunLZ and rFosLZ were obtained by cleaving fusion protein bound to GSH-agarose columns with thrombin (Riley *et al*, 1994). Note that thrombin cleavage of the fusion proteins leaves two vestigial residues from the thrombin recognition site (Gly1-Ser2) at the N-terminus of the recombinant peptides. Further purification of the peptides was achieved by reverse-phase HPLC chromatography using a linear acetonitrile gradient on a Pharmacia C_2/C_{18} Pep-S column (Pharmacia, Uppsala, Sweden) as described previously (Riley *et al*., 1994).

Protein Labeling

S-Carboxymethylation of Cysteine Residues. For all ELISAs, it was necessary to block the cysteine residues of rFosLZ-GST in order to prevent the formation of disulfide-linked covalent dimers in binding assays. Additionally, it was necessary to block the N-terminal cysteine residue of rJunLZ or rJunLZ-GST for ELISAs which employed lysine rather than cysteine biotinylation (see below). Cysteine modifications were performed as follows: solutions of rJunLZ (in 0.1 M $NaHCO_3$, pH 8.5) or rFosLZ-GST or rJunLZ-GST (in 50 mM Tris, pH 8.0, containing 5 mM GSH) were incubated for 1 h at ambient temperature in the presence of 50 mM dithiothreitol in order to reduce any disulfide bonds. IAA (85 mM) was then added, and the solutions were then incubated at ambient temperature for an additional 1 h to allow alkylation of the cysteine residues. Excess reagents were then removed from fusion protein solutions by dialyzing at 4 °C over a 72-h period against PBS buffer. IAA-labeled rJunLZ was biotinylated and purified by HPLC as described below.

Biotinylation. We examined two types of rJunLZ biotinylation for use in immunoassays: rJunLZ-GST, rJunLZ, and rJunLZ(V36E) were biotinylated using either the lysine-specific reagent biotin-XX-NHS (where X is an aminocaproyl spacer and NHS is *N*-hydroxysuccinimide) or the cysteine-specific reagent iodoacetyl-LC-biotin (where LC is a hexylenediamine spacer). For Lys biotinylation, a 4-fold molar excess of biotin-XX-NHS, dissolved in DMSO, was added to IAA-labeled protein (in 0.1 M $NaHCO_3$, pH 8.5), and the mixture was incubated for 2 h at ambient temperature. Unreacted biotin-XX-NHS was removed from rJunLZ-GST solutions by dialyzing against PBS (0.1 M Na_2HPO_4, 0.15 M NaCl, 3 mM NaN_3, pH 7.4) over a 72-h period. Biotinylated HRP (b-HRP) and biotinylated GαHRP (b-GαHRP) were prepared similarly using a 1:5 w/w biotin:protein ratio. Biotinylated rJunLZ (b-JunLZ) was purified away from labeling reagents by reverse-phase HPLC on a Pharmacia C_2/C_{18} column.

Biotinylation of the N-terminal cysteine residue of rJunLZ and rJunLZ(V36E) was achieved by adding to the reduced, lyophilized peptides a 5−10-fold molar excess of iodoacetyl-LC-biotin dissolved in a mixture of 20% DMSO and 80% 0.1 M sodium phosphate containing 10 mM EDTA. The pH was adjusted to 7.5, and the reaction was allowed to proceed for 2 h at 30 °C. Biotinylated peptides were purified away from labeling reagents by reverse-phase HPLC on a Pharmacia C_2/C_{18} column. The extent of biotinylation was monitored by mass spectrometry of the purified peptide. The terms bCys and bLys are used below to distinguish between rJunLZ biotinylated at cysteine or lysine residues.

Concentrations of biotinylated peptides were determined by automated amino acid analysis at the Macquarie University Centre for Analytical Biotechnology (Sydney, Australia).

Immunoassays

All ELISA experiments were performed using 96-well polystyrene trays purchased from Disposable Products (Adelaide, SA, Australia). rFosLZ-GST labeled with IAA was

adsorbed to the wells of an ELISA plate by incubation at 37 °C for 1 h, and the remaining binding capacity of the wells was eliminated by a 1-h incubation with 1% (w/v) heat-denatured casein (HDC) in PBS containing 2.7 mM thymol (HDC/PBS/thymol). b-rJunLZ or b-rJunLZ(V36E) was then applied to the plate, serially diluted in HDC/PBS/thymol, and allowed to dimerize via its LZ motif with plate-bound rFosLZ-GST by incubation at 37 °C for 1 h. Significant amplification and detection of this specific interaction was achieved with the subsequent addition of one to three layers of ECA solution (see below). This was followed by the addition of substrate solution [3−14 mM *o*-phenylenediamine in 0.05 M sodium citrate, 0.1 M NaH_2PO_4, pH 5, containing 0.03% (w/v) H_2O_2] in order to detect plate-bound HRP. After allowing sufficient time for color development (typically 3−5 min) the reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 490 nm using a Bio-Rad model 450 Microplate Reader. Color development is directly proportional to the concentration of bound HRP which in turn is directly proportional to the concentration of b-rJunLZ-GST, b-rJunLZ, or b-rJunLZ(V36E) bound to the ELISA plate.

Signal Amplification with ECA

Assembly of the ECA complex was achieved as described previously (Wilson & Easterbrook-Smith, 1993) by mixing SA and b-HRP (both in HDC/PBS/thymol) at a concentration of 2.0 μg/mL each. Following an incubation period of between 40 min and 2 h, b-GαHRP was added to 2.0 μg/mL, followed by an additional 2.0 μg of SA/mL 1 min later. 50-μL aliquots were then added immediately to the plate. To obtain increased signal amplification, three successive layers of ECA were specifically bound to antigen as follows. After the unbound initial-layer ECA complex had been washed away, ELISA wells were incubated with 2.0 μg of b-GαHRP/mL, and this was followed by an additional incubation with freshly prepared ECA complex. This process was repeated when required to yield three layers of specifically bound ECA complex (ECA-3).

Data Analysis. Individual estimates of dissociation constants (K_d) were obtained by nonlinear regression analysis of eq 5 (see below) onto ELISA-derived binding curves using KaleidaGraph 3.0 (Synergy Software, Reading, PA) with each datum weighted according to its standard deviation (SD). Each datum point and its associated SD was derived from triplicate lanes on a single ELISA plate. Weighted arithmetic means and grouped standard deviations were calculated according to the equations given in Spiegel (1972) using independent K_d estimates derived from a number of ELISA experiments performed on different trays. Simulations of the effects of ELISA washing procedures on measured dissociation constants were carried out using Mathematica 2.2 (Wolfram Research, Inc., Champaign, IL).

RESULTS

Expression and Purification of Fusion Proteins. Figure 1 summarizes the overexpression of rFosLZ-GST fusion protein from the sequenced clone (DH5α/pFLZ). There was minimal heterologous protein expression by cells which were not treated with IPTG (Figure 1, lane 2), whereas rFosLZ-GST represented ∼20% of soluble cell protein in cells treated with 75 μM IPTG at 32 °C (Figure 1, lane 3). The fusion protein was present almost exclusively in the soluble fraction

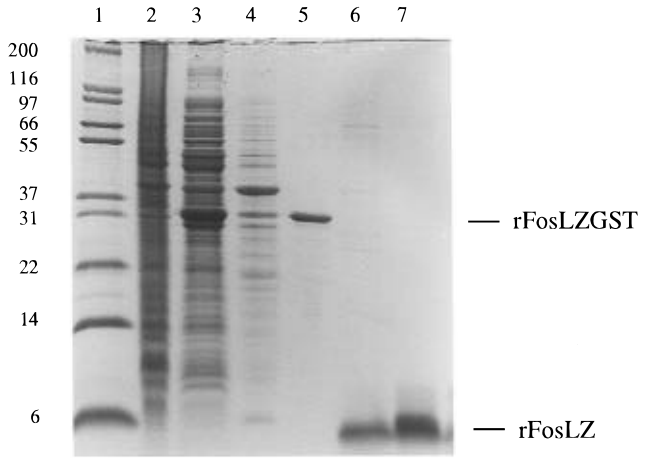

FIGURE 1: Discontinuous SDS−PAGE gel (Schägger & von Jagow, 1987) illustrating the overexpression and purification of rFosLZ as a fusion protein or LZ peptide. Lane 1, standard molecular mass markers (size is given in kDa on the left of the gel); lane 2, total protein profile from uninduced *E. coli* DH5α/pFLZ; lane 3, soluble cell protein following induction; lane 4, insoluble cell protein following induction; lane 5, glutathione affinity-purified rFosLZ-GST; lane 6, glutathione affinity-purified rFosLZ; lane 7, rFosLZ after HPLC purification.

of the cell lysate (compare lanes 3 and 4, Figure 1), and was readily purified using *S*-hexylglutathione agarose affinity chromatography (Figure 1, lane 5). rJunLZ-GST was produced and purified in a similar manner (Riley *et al*., 1994). Typically more than 50 mg of the fusion proteins was recovered per liter of cell culture.

Purification of rFosLZ and rJunLZ. We showed previously that it is possible to efficiently purify rJunLZ by subjecting the column eluate from thrombin digests of GSH-agarose-bound rJunLZ-GST to C_2/C_{18} reverse-phase HPLC (Riley *et al*., 1994). A similar procedure was used to obtain rFosLZ. Treatment of GSH-agarose-bound rFosLZ-GST with thrombin released a species with an apparent molecular weight (MW) of ∼5 kDa on discontinuous SDS−PAGE gels (Figure 1, lane 6), consistent with the expected mass of an rFosLZ monomer. The identity of the HPLC-purified peptide (Figure 1, lane 7) was confirmed by electrospray mass spectrometry (calculated MW = 5059.6, experimental MW = 5061.3).

ECA Allows Detection of Subnanomolar Concentrations of Biotinylated Leucine Zipper Dimers. There are two reported estimates of the dissociation constant for the dimer of native Jun and Fos LZ domains. Pernelle *et al*. (1993) reported a value of 110 ± 12 nM, while a lower estimate of 23 ± 9 nM was obtained by Patel *et al*. (1994). In addition, dissociation constants as low as 4 pM have been reported for model leucine zipper peptides (O'Neil *et al*., 1990).

Thus, it was necessary to develop ELISA methods with sufficient sensitivity to allow detection of subnanomolar concentrations of Jun and Fos. This was done using ECA. In this procedure, complexes containing the ELISA detection enzyme (in this case horseradish peroxidase) are formed through reactions involving streptavidin, biotinylated detection enzyme, and a biotinylated polyclonal antibody specific for the detection enzyme. These complexes can then be used to detect biotinylated analytes. In model systems, the ECA procedure has been shown to give a gain in sensitivity approaching 3 orders of magnitude over that achieved using complexes of the detection enzyme and streptavidin only (Wilson & Easterbrook-Smith, 1993). One advantage of the

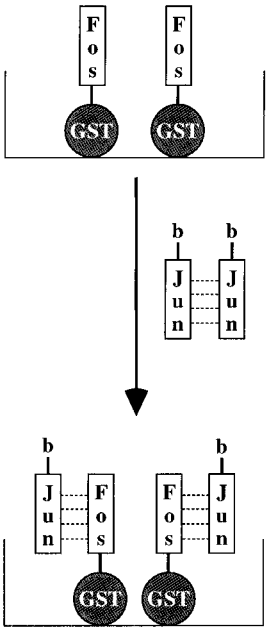

FIGURE 2: Cartoon of the peptide-based ELISA used for detection of Jun and Fos. This scheme is based on the ability of biotinylated rJunLZ to form a leucine zipper with rFosLZ-GST bound to an ELISA plate. The biotinylated Jun species in the heterodimeric complex can then be detected with ECA complexes (see text). Note that heterodimer formation is much preferred over homodimer formation due to the relative instability of the Fos homodimer ($K_d \approx 6$ μM; O'Shea *et al*., 1989).

ECA procedure is that is it possible to increase the sensitivity of an ELISA by adding additional aliquots of the ECA reagent after the first aliquot has been allowed to react with the target analyte. In principle, this procedure can be continued indefinitely, giving ELISA protocols with one (ECA-1), two (ECA-2), three (ECA-3), or more "layers" of the ECA reagent (Wilson & Easterbrook-Smith, 1993).

The strategy employed in the ELISA experiments is outlined schematically in Figure 2. ELISA trays were initially coated with rFosLZ-GST fusion protein and then blocked with HDC prior to addition of biotinylated JunLZ. In order to test the sensitivity of the ECA method relative to conventional SA-HRP conjugation, bLys-rJunLZ-GST fusion protein was dissolved in HDC and added to ELISA plates at various concentrations up to 10 nM. Complexes of this species with rFosLZ-GST were detected using either a commercially available SA-HRP conjugate or ECA. The data in Figure 3 show that, while the commercial SA-HRP conjugate was unable to detect complexes of bLys-rJunLZ-GST and rFosLZ-GST when the input concentration of b-rJunLZ-GST was less than 10 nM, detection of such complexes was possible using ECA. Moreover, use of increasing "layers" of ECA reagent gave progressive increases in the specific ELISA signals; the ECA-3 signal was still 2.3-fold greater than the background at a bLys-rJunLZ-GST concentration of 0.3 nM.

The substrate concentration used for the experiments shown in Figure 3 was chosen to permit sensible comparisons between ECA-1, ECA-2, ECA-3, and commerical SA-HRP conjugate. However, substantially lower concentrations of rJunLZ could be detected by using higher substrate concentrations in combination with ECA-2 or ECA-3. For example, we showed previously that BSA concentrations as low as 7.5 pM could be detected using ECA-3 (Wilson & Easterbrook-Smith, 1993). In the present case, the detection limit

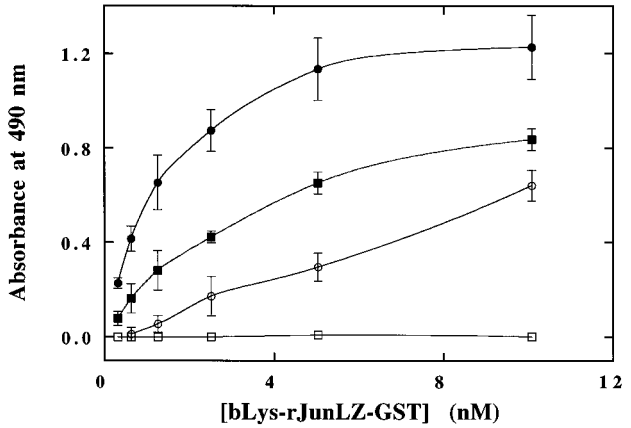

FIGURE 3: Comparison of the ELISA signal generated by SA-HRP, ECA-1, ECA-2, and ECA-3 in detection of plate-bound Fos. rFosLZ-GST was applied to an ELISA plate at a concentration of 1.6 μM and allowed to adsorb to the well surfaces by incubation at 37 °C for 1 h. The remaining binding sites on the ELISA plate were blocked by incubation for 1 h at 37 °C with HDC/PBS/thymol, and then b-rJunLZ-GST was applied to the plate at a concentration of 161 nM in HDC/PBS/thymol and serially titrated 1:2 in this buffer. After a 1-h incubation at 37 °C, detection of bound b-rJunLZ-GST was achieved by additional 1-h incubations at 37 °C using either SA-HRP conjugate (□), ECA-1 (○), ECA-2 (■), or ECA-3 (●), followed by addition of substrate solution. Data are the means ± SD of triplicates.

is set largely by the K_d for rJunLZ:rFosLZ complex formation (see below), rather than the intrinsic sensitivity of the ECA method, as it is impossible to "capture" significant amounts of b-rJunLZ at input concentrations well below the dissociation constant.

Peptide-Based ELISA Can Be Used To Measure Dissociation Constants. Data of the type shown in Figure 3 may be used to extract dissociation constants for the Jun:Fos interaction. Figure 4A illustrates the titration of plate-bound rFosLZ-GST with either bCys-rJunLZ or bLys-rJunLZ peptide. These data were analyzed by considering that the binding of b-rJunLZ to rFosLZ-GST adsorbed to the wells of an ELISA tray can be described by the equilibrium constant (K_d)

$$K_d = \frac{[J][F]}{[JF]} \quad (1)$$

where [J] is the concentration of free b-rJunLZ, [F] is the concentration of plate-bound rFosLZ-GST which is not complexed with Jun, and [JF] is the concentration of b-rJunLZ complexed to plate-bound rFosLZ-GST. From eq 1 and mass conservation eqs 2 and 3

$$J_{tot} = [J] + [JF] \quad (2)$$

$$F_{tot} = [F] + [JF] \quad (3)$$

[JF] can be calculated by solving the following quadratic:

$$J_{tot}F_{tot} - (K_d + F_{tot} + J_{tot})[JF] + [JF]^2 = 0 \quad (4)$$

Nonlinear least-squares analysis of the data shown in Figure 4 can now be carried out according to eq 5:

$$A = \alpha[JF] + \beta \quad (5)$$

where A is the measured ELISA signal, α is the effective molar absorptivity (L mol^{-1}), and β is a term that allows for loss of signal during washing (see below). The least-squares analysis was carried out by fitting four parameters, namely,

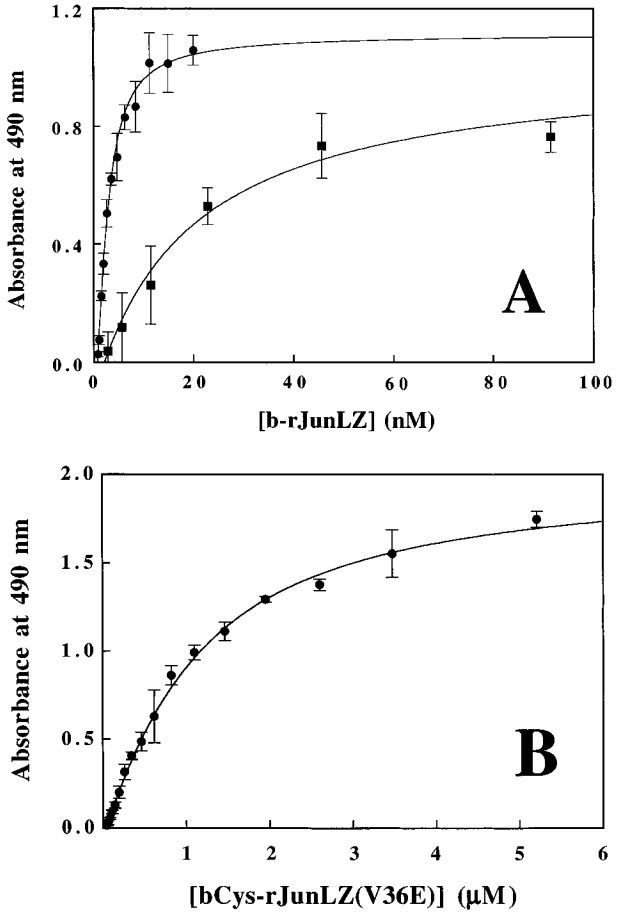

FIGURE 4: Measurement of Jun:Fos dissociation constants. rFosLZ-GST was applied to the ELISA plate at a concentration of 0.32 μM, and the remaining protein binding sites were blocked by a 1-h incubation with HDC/PBS/thymol. (A) bLys-rJunLZ (■) or bCys-rJunLZ (●) was then applied to the plate at a concentration of 1.5 μM or 20 nM, respectively, followed by serial dilution and detection using ECA-1. Data are the means ± SD of triplicates. The two curves have been normalized so that they have the same maximum absorbance. (B) bCys-rJunLZ was applied to the plate at a concentration of 5.2 μM, serially diluted, and detected using ECA-1. The solid lines in both figures represent the best fit of eq 5 to the data, which yielded K_d values of 0.98 nM, 19 nM, and 0.82 μM for bCys-rJunLZ, bLys-rJunLZ, and bCys-rJunLZ(V36E), respectively.

K_d, α, β, and F_{tot}. Thus it is not necessary to know *a priori* the concentration of plate-bound rFosLZ-GST, which will depend on a number of factors, including the concentration of the rFosLZ-GST solution initially applied to the ELISA plate and the affinity of the GST moiety for the plate surface. The data shown as circles in Figure 4A represent a typical titration curve (derived from triplicates on a single ELISA plate) for the reaction of bCys-rJunLZ with plate-bound rFosLZ-GST. Fitting of eq 5 to this data yielded a K_d of 0.98 nM. Analyses of five similar data sets gave a mean dissociation constant of 0.99 ± 0.30 nM.

Figure 4B shows a typical titration curve (derived from triplicates on a single ELISA plate) obtained using bCys-rJunLZ(V36E). Clearly, the higher scale on the abscissa indicates that the mutant peptide has a much lower affinity for plate-bound FosLZ-GST. Fitting of eq 5 to this data yielded a K_d of 0.82 μM; analyses of two independent data sets gave a mean dissociation constant of 0.90 ± 0.13 μM.

Possible Influence of Biotinylation on K_d for Zipper Formation. In order to investigate the possibility that the derivatisation of the LZ peptides with biotin might significantly alter their dimerization affinities, we carried out immunoassays using either lysine- or cysteine-biotinylated peptides. Since the only cysteine residue in these peptides is located N-terminal to the LZ region, it seems unlikely that biotinylation at this position would significantly affect dimerization affinity. However, while the lysine residues in rJunLZ are not involved directly in the hydrophobic coiled-coil interface (Glover & Harrison, 1995; Junius *et al*., 1996), their extended hydrophobic side chains do flank the dimer interface and their terminal charged amine groups are involved in favorable interhelical electrostatic interactions with Glu residues of Fos in the crystal structure of the Jun-bZIP/Fos-bZIP/AP-1 complex (Glover & Harrison, 1995).

A comparison of the individual titration curves obtained for bLys-rJunLZ and bCys-rJunLZ is shown in Figure 4A (squares and circles, respectively). It is clear from visual inspection of these curves that dimerization affinity is significantly reduced by incorporation of biotin at lysine residues. This was confirmed by quantitative analysis of data sets from four independent ELISA plates which indicated that the K_d for bLys-rJunLZ (18 ± 4 nM) was approximately 20-fold higher than the value obtained using bCys-rJunLZ.

Effect of Plate-Washing on K_d Measurements. Before the values for the equilibrium constants for Jun:Fos dimerization obtained in this study could be compared with those estimated from other experimental approaches, it was necessary to consider the possible effects of details of the ELISA protocol on the derived parameters. Specifically, after the b-rJunLZ ligand had been allowed to reach equilibrium with plate-bound rFosLZ-GST, excess (unbound) reagent was removed by washing. This step was followed by addition of one to three layers of ECA reagent, with wash steps after addition of each layer of this reagent. At each of these wash steps, some of the biotinylated species may be washed from the plate, giving rise to a reduced ELISA signal.

In general terms, there are potentially three ways in which the shape of the binding curve could be affected by the washing procedure. First, the removal of a constant *amount* of biotinylated species (independent of added rJunLZ concentration) would shift the whole binding curve in the $-y$ direction, while retaining the same shape. This corresponds to a negative value of β in eq 5 and a negative y-intercept for the curve. Thus, a curve of the shape shown in Figure 5 (dotted line) would be obtained, although the portion of the curve corresponding to negative absorbance values would be experimentally inaccessible. In the second possibility, a constant *proportion* of the biotinylated species may be washed off, giving a binding curve of the type shown as a dashed line in Figure 5; this corresponds to an altered value of α in eq 5, which would not affect the measured K_d. Finally, an amount of rJunLZ could be removed which is dependent both on the concentration of rJunLZ present and its affinity for the plate-bound rFosLZ-GST (simulated curve not shown). In this case, the measured binding affinity would be an underestimate the actual value.

Of these three possibilities, our experimental data is, in all cases, best represented by the first model, giving rise to negative values for the parameter β in the experimental curve fits. Thus, points for which the measured absorbance was not significantly different from zero were excluded from the curve fitting procedure. The lack of dependence of the washing behavior on rJunLZ concentration may suggest that it is in fact the plate-bound rFosLZ-GST which is washed off.

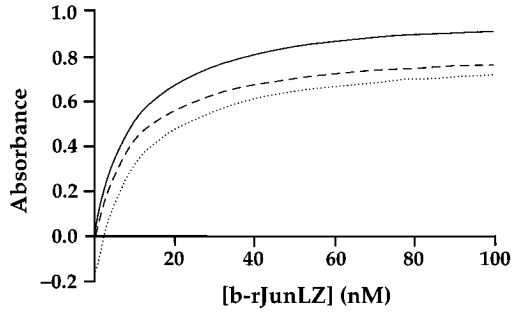

FIGURE 5: Simulation of the effects of washing on measured dissociation constants. The solid line represents the binding curve obtained if washing effects are neglected (assuming $\alpha = 5 \times 10^{10}$ M^{-1}, $\beta = 0$, $K_d = 10$ nM, and $F_{tot} = 2 \times 10^{-11}$ M). The dashed line represents the curve obtained if a constant *proportion* (one-sixth) of biotinylated species is removed during the washing procedure; this corresponds to a decrease in the value of α in eq 5. The dotted line represents the binding curve obtained if a constant *amount* of biotinylated species (corresponding to an absorbance of 0.2) is removed during washing; this corresponds to a negative value of β (-0.2) in eq 5.

DISCUSSION

Measurement of LZ Dimerization Affinities. To date, estimates of the dissociation constant for the Jun:Fos heterodimer have been made using three experimental approaches: use of the radioactivity of ^{125}I-labeled FosLZ to induce fluorescence when this ligand was found to JunLZ attached to fluorophore-impregnated microspheres (Pernelle *et al*., 1993), fluorescence resonance energy transfer between fluorescein-labeled Fos bZIP domain and rhodamine-labeled Jun bZIP domain (Patel *et al*., 1994), and ELISA measurements using biotinylated JunLZ (this study). These different approaches have yielded respective estimates of the K_d for Jun:Fos dimerization of 110 ± 12, 23 ± 9, and 0.99 ± 0.30 nM. One potential limitation of the experimental design adopted in this study is that our estimate of the K_d is based on the binding of biotinylated rJunLZ to rFosLZ-GST immobilized on the surface of wells of ELISA trays. It is thus possible that the steric constraints arising from the adsorption of rFosLZ-GST to ELISA trays may have led to systematic errors in the analysis of the binding of biotinylated Jun to this immobilized ligand. Although this possibility cannot be discounted, the observation that our estimate of the K_d is significantly *lower* that obtained in the other studies makes it unlikely that there is a significant steric impediment to dimerization in this system.

There are a number of possible explanations for the large discordance between the estimated K_d values obtained by Patel *et al*. (1994) and in this study and the value of 110 nM reported by Pernelle *et al*. (1993). Patel and co-workers suggested that one contribution to the discordance may have been that the recombinant peptides used by Pernelle *et al*. (1993) did not include the His residues found in phase with the leucines, C-terminal to the leucine zippers; it has been proposed that these His residues may be important in dimer formation (Cohen & Curran, 1990). However, our estimate of the K_d value was obtained from experiments using recombinant peptides which did not contain these His residues, suggesting that they do not play a significant role in determining dimerization affinity.

An alternative explanation for the discrepancy may be that Pernelle *et al*. (1993) measured formation of Jun:Fos heterodimers using a buffer containing 0.1% Triton X-100 (approximately 1.5 mM detergent). While it was shown that 0.1% Triton X-100 had no effect on the melting temperature of Jun:Fos heterodimers, we note that the thermal denaturation experiments were carried out using 50 μM Jun:Fos heterodimer (giving a molar detergent:protein ratio of approximately 30:1), whereas far lower protein concentrations were used in the scintillation proximity assays used to measure the dissociation constants (giving detergent:protein ratios of 3000:1 or more). At these high detergent:protein ratios, the Triton X-100 may partition more effectively onto the hydrophobic faces of the amphipathic α-helices of FosLZ and JunLZ. This might be expected to compromise their ability to form heterodimers, perhaps accounting for the relatively high K_d values obtained by Pernelle *et al*. (1993); however, we obtained no significant change in the K_d values we measured when 0.1% Triton X-100 was co-added with b-rJunLZ in the ELISA protocol (data not shown).

The difference between the K_d value we measured and that reported by Patel *et al*. (1994) probably reflects an intrinsic difference in the dimerization capabilities of the isolated LZ domain (used in our experiments) and the extended bZIP motif (used by Patel and co-workers). The 22-residue DNA-binding motifs of the bZIP domain are extremely basic, with a net charge of +10 for both Jun and Fos. When not interacting with DNA, these unstructured basic domains protrude from the leucine zipper and most likely provide a significant repulsive electrostatic force, thereby lowering dimerization affinity.

An important caveat to note for the ELISA described herein and any binding assay employing covalently modified molecules is that one must try if possible to assess the effect of the modification on the measured binding constants. In the current case, it was found that biotinylation at Lys residues located in the LZ domain gave 20-fold reduced dimerization affinities compared with peptides that had been biotinylated at an N-terminal Cys residue located outside the dimerization interface. The crystal structure of the Jun-bZIP/Fos-bZIP/AP-1 complex (Glover & Harrison, 1995) reveals three interhelical ion pairs between Lys residues on c-Jun and Glu residues on c-Fos. These electrostatic interactions are thought to contribute favorably to LZ stability (Zhou *et al*., 1994), and hence abrogation of these interactions through Lys biotinylation, together with potentially unfavorable steric effects from the bulky XX-biotin moeity, most likely accounts for the reduced dimerization affinities.

Screening for Dominant-Negative LZ Domains. A number of proteins from the bZIP and bHLH-ZIP families of transcription factors (where the latter family of proteins contains an additional helix−turn−helix motif interposed between the LZ and basic domains) are thought to be intimately involved in the neoplastic transformation of cells; in particular, Jun, Fos, and Myc have been implicated in the development of a number of different types of cancers (Riou *et al*., 1990; Field & Spandidos, 1990; Morgenbesser & DePinho, 1994; Angel & Herrlich, 1994). Thus, molecules that inhibit the dimerization of bZIP and bHLH-ZIP proteins might be effective therapeutic agents as they would restrict the transcriptional regulatory activity of these proteins. Dominant-negative Jun mutants have been used to inhibit a number of biological phenomena, including apoptosis of sympathetic neurons following withdrawal of nerve growth factor (Ham *et al*., 1995), the transcriptional activity of nuclear factor AT (NF-AT) in lymphocytes (Petrak *et al*., 1994), and ras-mediated cellular transformation (Lloyd *et al*., 1991; Granger-Schnarr *et al*., 1992; Brown *et al*., 1993).

Fos and Jun LZ peptides also effectively suppress insulin-mediated maturation when injected into oocytes (Pernelle *et al*., 1993). Thus, in order to test whether the peptide-based ELISA could readily discriminate between engineered LZ domains with different dimerization affinities, we compared the dimerization affinity of native JunLZ with that of a V36E point mutant. These experiments (see Figure 4) revealed that the V36E mutation substantially destabilizes the Jun:Fos heterodimeric leucine zipper.

The rJunLZ(V36E) mutant was originally designed with the idea of forming a Glu−Lys salt bridge at the interface of the heterodimer in order to increase its stability. However, the reduced dimerization affinity can be explained by examining the known conformational preferences of residues in various positions of LZ coiled-coils. Following the standard nomenclature for coiled-coils [(**abcdefg**)$_n$; McLachlan & Stewart, 1975], the dimer interface of leucine zippers is comprised of alternating rungs of **d**-position Leu residues and apolar **a**-position residues which generally have β-branched side chains. The three-dimensional structures of the GCN4 (O'Shea *et al*., 1991) and c-Jun LZ (Junius *et al*., 1996) domains reveal that the **d**-position residues pack in a so-called "perpendicular" orientation (Harbury *et al*., 1993) such that they point into the dimer interface and make extensive van der Waals contacts. On the other hand, **a**-position residues pack in a "parallel" orientation (Harbury *et al*., 1993) such that they point away from the dimer interface into the bulk solvent. Glu 36 in the V36E mutant resides in an **a**-position, and therefore its side chain carboxylate group would be placed at a considerable distance from the amine group of the adjacent **a**-position Lys side chain contributed by c-Fos, thus precluding the formation of an ion pair. The reduced dimerization affinity is therefore likely to be a consequence of the reduced hydrophobic interaction associated with the V36E substitution not being compensated for by the formation of an interhelical Glu−Lys ion pair. However, even if was geometrically possible to form a Lys−Glu ion pair, the mutant structure might not be as stable as the native heterodimer in which the terminal amine group of the Lys residue forms a hydrogen bond with the side chain carbonyl oxygen of a neighboring **g**-position Gln in Jun (Glover & Harrison, 1995).

These results demonstrate the utility of the novel peptide-based ELISA described herein for characterizing the effects of point mutations on LZ dimerization affinity. The assay can be extended to any pair of interacting LZ proteins, and hence it could be used to rapidly screen for molecules that are capable of inhibiting the activity of bZIP and bHLH-ZIP proteins. At a more fundamental level, the availability of a facile assay for leucine zipper formation will allow a dissection of the precise molecular contributions to the affinity and specificity of this important protein dimerization motif.

ACKNOWLEDGMENT

The authors thank Dr. Lindsey Mackay (Australian Government Analytical Laboratories, Sydney) for mass spectrometric analyses and Ms. Lisa Riley for stimulating discussions. We also thank the reviewers of this manuscript for helpful comments.

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BI952817O